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16. ABSTRACT

Many toxicologists in academic, industrial and governmental sectors consider in vitro (i.e. cell and tissue culture) alternatives to animal toxicity testing as inevitable given the sheer volume of new chemicals and the time and cost confines of conventional methodologies (Roofe, 1971; Nardone, 1977; Tardiff, 1978; Stammatti et al., 1981; Ekwall, 1983; Goldberg, 1984; Grisham and Smith, 1984). For Neurotoxicologists, in vitro systems ranging from tumor cell lines (i.e., neuroblastomas, gliomas, and pheochromocytomas) to organotypic explants provide rapid, inexpensive models for either screening putative neurotoxicants or addressing their mechanisms(s) of action. Ideally, a variety of methodologies are available to the investigator, since no single technique will be able to detect all possible neurotoxic end-points. Assuming this accessibility, a chemical's toxic potential can best be addressed using a tier approach in which neurotoxic assessment progresses from the simple, inexpensive and less specific assay (e.g., cell lines) to the more complex, technically demanding and definitive assay (e.g., tissue explant). The first tier acts as the preliminary screen for putative neurotoxicants and comments on a chemical's non-specific toxicity, usually in terms of cell viability. The next level of inquiry addresses the pathogenesis or mechanisms of injury and is designed to describe neurotoxicity in morphological, biochemical, and in some cases, electrophysiological terms.

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Many toxicologists in academic, industrial and governmental sectors consider in vitro (i.e. cell and tissue culture) alternatives to animal toxicity testing as inevitable given the sheer volume of new chemicals and the time and cost confines of conventional methodologies (Roofe, 1971; Nardone, 1977; Tardiff, 1978; Stammatti et al., 1981; Ekwall, 1983; Goldberg, 1984; Grisham and Smith, 1984). For Neurotoxicologists, in vitro systems ranging from tumor cell lines (i.e., neuroblastomas, gliomas, and pheochromocytomas) to organotypic explants provide rapid, inexpensive models for either screening putative neurotoxicants or addressing their mechanism(s) of action. Ideally, a variety of methodologies are available to the investigator, since no single technique will be able to detect all possible neurotoxic end-points. Assuming this accessibility, a chemical's toxic potential can best be addressed using a tier approach in which neurotoxic assessment progresses from the simple, inexpensive and less specific assay (e.g., cell lines) to the more complex, technically demanding and definitive assay (e.g., tissue explant). The first tier acts as the preliminary screen for putative neurotoxicants and comments on a chemical's non-specific toxicity, usually in terms of cell viability. The next level of inquiry addresses the pathogenesis or mechanisms of injury and is designed to describe neurotoxicity in morphological, biochemical, and in some cases, electrophysiological terms. With this methodology, care is taken to preserve the unique structural and biochemical properties of nervous tissue (see chapter IV) so that these features might be exploited as experimental end-points. At this level, systems are used that can detect the major target sites of neurotoxicity (i.e., neuronopathy, myelinopathy, and axonopathy), (Spencer and Schaumburg, 1980). This type of in vitro system is decidely more intact and differentiated from that used in

screening and is profitably combined with biochemical or pharmacological measurements. A further level of neurotoxic inquire can be designed to appraise chemical-risk. For this, a system that employs a statistically sufficient population and is amenable to pharmacokinetics and multi-dose testing is used. For such studies, experimental design should be based on a thorough knowledge of the metabolism, kinetics, and biology of the chemical to be evaluated.

Many advantages exist to using cultured materials over intact animals in neurotoxicology. Test chemicals, dissolved in the feeding media, come into direct tissue contact without filtration by the blood-brain barrier and with minimal lipid-aquaeous partitioning. Because the chemicals are dissolved in the nutrient fluids, toxic-exposure is continuous and easily quantitated, providing a means to manipulate chemically the tissue's mileu. Systemic effects, imposed by other organs (e.g., immune, endocrine, digestive, vascular, renal), which can interact with and compound a chemical's primary effect, are also by-passed. Systems can be chosen which employ chemically-defined nutrient media, thereby eliminating hepatic influences (i.e., mixed-function-oxidation, phase II conjugation) and afford a means to assess a chemical's "intrinsic" neurotoxicity. Alternatively, microsomal additives in the form of embryo extract can be added to the feeding media, imparting a limited metabolic competence to certain tissue culture systems. To facilitate data extrapolation, the basic design of an _in vitro_ neurotoxicological experiment should relate to clinical findings or contemporaneous animal studies with regard to dosing parameters, target tissue, and neuropathic end-points. Since a variety of nerve culture systems exists, judicious selection of an appropriate model is critical. For example, if the neurotoxic potency of a particular test chemical relies on

hepatic conversion, data gleaned from a culture system which uses a chemically defined media will be meaningless when related to whole animal studies. Alternatively, if a chemical's intrinsic toxicity is at issue, one is limited to using cells or tissues which can grow in chemically defined media. Likewise, if secondary phenomena such as lipid-aquaeous partitioning are unique controlling mechanisms in the bioavailability of a test chemical, a more differentiated system (viz. myelinated nerve fibers as opposed to isolated cells) should be used.

The successful culturing of nerve cells and tissues requires living nerve tissues to continue normal structural, biochemical and physiological differentiation in spite of being isolated from the body; an ambitious undertaking that demands a unique level of technical expertise. Sterile techniques are fundamental to all procedures since explanted tissues can no longer rely on the immune system. In some of the less differentiated in vitro systems (e.g. cell lines), antibiotics are routinely incorporated into the feeding media, in contrast to fetal tissue explants. Careful control of the bathing and feeding solutions are vital to both the viability of the system and the valid interpretation of the results. The bathing solutions are usually made up of salts, amino acids, and ions, in proportions devised to correct for osmotic pressure and to maintain an optimal pH. Ingredients contained in the nutrient media include nerve growth factor, fetal calf serum and human placental serum which contain a variety of hormones and proteins. The role of blood serum, another ingredient of the nutrient media, is not entirely understood but appears to provide necessary growth factors for the cultured tissues. Dialized rat-tail collagen (Bornstein, 1958; Masurovsky and Peterson, 1973) or commercial substrates such as poly-lysine provide a suitable substrate for the neuritic outgrowth of

explanted tissue fragments and dissociated cells. In vitro housing-chambers vary in shape and material depending on the methodology. For short-term cell cultures, plastic petri dishes or flasks may be used, but for long-term cultures, glass containers, free of silicon impurities, are necessary. Numerous pitfalls are encountered when working with cultured nerve tissues. Those systems that require the use of fetal calf serum or human placental serum in their nutrient media pose distinct problems since the explanted or dissociated neurons are not protected by a blood-brain barrier and are exposed to a variety of unknown drugs and metabolites present in these fluids. The housing chambers of cultured tissues, be they petri dishes or flasks, allow the metabolic production of CO_2 to accumulate in the nutrient fluid, contributing to pH shifts in the tissue's mileu, which can contribute to the variable quality sometimes encountered in nerve cultures.

In the following section various in vitro systems that have be used in neurotoxicology will be described. Other methodologies, not discussed, notably whole organ and embryo cultures, are amenable to assessing neurotoxic chemicals, but have yet to be exploited. For a more comprehensive discussion of the use of tissue culture in neurotoxicology several review articles are referenced (Goldberg, 1980; Goldberg et al., 1980; Yonezawa et al., 1980; Schrier, 1982)

Cell Lines: Neural or glial tumor cell lines are ideal screening systems since large numbers of homogeneous cell types can be cultured for biochemical and morphological assessment. Cell lines are isolated from the normal cytoarchitecture and extracellular matrices of intact nervous tissue and consequently cannot mimic the complex interactions of the various cell types in a nerve fiber, no matter how exquisite the experimental design. Cell lines are relatively static systems in which the dose of the test

chemical and the duration of contact does not resemble that which occurs *in vivo*. Because of these factors, the limitation of this technique as a chemical screening tool to collect base-line data is recognized in neurotoxicological research.

The mouse neuroblastoma cell is the most popular tumor cell-line used in neurotoxicolgy. These cells, derived from a neoplasma over 40 years ago, are propagated *in vitro* by subcutaneous transfer. Because they form homogenous populations which proliferate rapidly in chemically defined media, extend neuritic-like processes and contain neurotransmitter precursor molecules (e.g., choline acetyltransferase and tyrosine hydroxylase) this cell line has been used extensively in neurotoxicology (Goldstein, 1977; Selkoe et al, 1978; Fedalei and Nardone, 1983). Criteria used to determine neurotoxicity are varied and include cell death, loss of viability, and impaired neuritic extension. In general, however, many investigators are reluctant to use a tumor cell line to comment on a chemical's neurotoxicity. Other disadvantages include variations in the response of different cell lines to chemical-exposure (Schrier, 1982). In addition, considerable difficulty has been reported in distinguishing between morphologically "differentiated" neuroblastomas and those showing a neurotoxic effect (Erkell and Walum, 1979). Glial-tumor cell lines (i.e., astrocytomas, Schwanomas) also are available but have yet to be used extensively in neurotoxicological research (Wennersten, 1979; Parmentier et al., 1978).

Reaggregated cell cultures: The technique of reaggregated cell culture lends itself especially well to coordinated morphological, and biochemical assessments of neurotoxicity (Trapp and Richelson, 1980). This technique, first developed by Moscona (1961), involves the enzymatic or mechanical dissociation of whole brain or brain parts into single cells. The cell

suspensions are held in plastic flasks or bottles, and under optimal conditions, migrate and organize themselves into suspended clusters which cytoarchitecturally resemble tissue from the sampled brain area (Sheffield and Moscona, 1970; DeLong, 1970; Levitt et al., 1976; Trapp, et al., 1979). The cells interact by cell-cell contacts and form three dimensional aggregates. Because they are grown in large rotated flasks, this system provides high cell yields, permitting standard biochemical analyses of the cells and tissues. A disadvantage of this technique is that microscopic observation, easily done with explants or monolayer cultures, cannot be performed due to the thickness (100-1000 um) of the aggregated cultures.

Dissociated Ganglia Cell Techniques: This technique uses dorsal root ganglia (DRG) or superior cervical ganglia as the tissue source. Although it has been widely used in developmental neurobiology, it is easily adapted to address neurotoxic mechanisms. Fetal material is either mechanically triturated by forcing the minced brain or ganglia through a 135 um nylon gauze net or enzymatically dissociated with trypsin or dispase. The single cell suspension is plated on collagen-coated cover-slips or plastic dishes and fed with nutrient fluids. At maturation, the dissociated cells form a monolayer with differentiated, myelinated nerve fibers. Although the three dimensional structure is lost, permitting interpretation only on a cellular level, this type of neuronal cell culture retains a rather impressive morphology in spite of its dissociation and affords parallel evaluation of morphological, biochemical (using microtechniques) and electrophysiological parameters.

Organotypic Explants: Cultured fragments of fetal central nervous (CNS) tissues, which preserve their three dimensional cytoarchitecture are termed "organotypic" explants and represent a unique link between the less

differentiated cell cultures and the intact animal. The methodology, first pioneered by Peterson and Murray (1955) and others (Pomerat and Costero, 1956; Hild, 1957) for developmental studies, was later used by electrophysiologists (Crain, 1964,1966) and neuropathologists (Bornstein, 1969; Raine, 1973). Many studies have since validated the morphological and bioelectrical parallels of the organotypic system to the in vivo state (Bunge et al 1965,1967; Crain et al., 1968; Sobkowicz et al., 1968; Peterson and Crain, 1965,1970). The most compelling feature of this technique is the possibility to explant different levels of the CNS neuraxis, either individually or in combination, and to observe microscopically their response to experimental manipulation. Nervous tissues that have been successfully cultured include olfactory bulbs, retina, optic tectum, cerebellum, cortex, medulla, spinal cord and peripheral ganglia (Yonezawa et al., 1980), a variety that permits one to address specific regional vulnerability. Although the explants can be housed in well-depression slides known as Maximow chambers (Maximow, 1925), Rose chambers, or special plastic cups known as Bunge dishes (Bunge and Wood, 1974), the Maximow chamber is designed, for microscopic monitoring of the progression of neuropathologic changes.

The procedures of the organotypic culture technique are exceptionally involved and rely on the technical expertise of various support groups. A brief description of these techniques follows but more detailed reviews are referenced (Bornstein, 1973; Yonezawa et al., 1980).

Glassware: Long-term organotypic culture requires the use of high quality glassware with low silicone impurities. The major stock glassware includes round 22mm, No. 1 thickness coverslips and 40mm No. 3 thickness squares. Goldseal Brand (Clay-Adams, Cat. No. A-1420) is chosen because

of its historical success in supporting long-term organotypic culture. The Maximow chambers are made from plate glass and measure 75 x 45 x 7mm. The cleaning and sterilization of the intimate glassware require repeated soakings in reagent grade 70% nitric acid, deionized water, and in 1% HCL. After further rinses with hot deionized water, the glassware is dried in 250°C ovens to burn off any remaining organic material. The glassware is then packaged for sterilization by double wraping in aluminum foil and heat-resistant paper.

Solutions and Media: Several solutions and media are routinely used in the maintenance of organtypic cultures: Hank's balanced salt solution (BSS), Eagle's Minimum Essential Medium (MEM), horse and calf serums, human placental serum, chick embryo extract and collagen. Both salt mixtures (BSS, MEM) can be commercially obtained (Flow Laboratories, NY). Human placental serum is obtained from blood removed from the placenta's umbilical vein immediately after delivery. The blood is carefully syringed into test tubes, taking care to avoid hemolysis, and refrigerated overnight. The serum which has separated from the clot is then partitioned into capped test tubes and refrigerated until use. Chick embryo extract is prepared from homogenized chick embryos removed from 9-day-old fertilized eggs under sterile conditions.

Collagen: Explanted tissues require a collagen-substrate to extend neuritic processes. The substrate is prepared from the tail-tendons of 400 to 500 g Sprague-Dawley rats which are removed by fracturing the tip of the tail with a Rongeux instrument (Roboz, Inc.) and pulling the collagen filaments out from the tail-base. The collagen strands are cut loose, collected in sterile distilled water, and transferred to an acetic acid solution, after which they are centrifuged. The collagen-acetic acid supernatant is

dialyzed for 24 hours at 4°C before the thickened collagen solution is refrigerated. To prepare the collagen-coated coverslips, the dialyzed collagen solution is mixed in a 9:1 ratio with 0.2% riboflavin. One drop of this solution is spread with a glass rod on a round coverslip and polymerized with incandescent lighting. The collagen-coated cover slips are then stored at room temperature in a staining jar containing BSS, glucose, fetal calf serum (which promotes the hydrophilic properties of the collagen gel surface), 1 drop of achromycin, 2 drops of gentamycin and 5 drops of penicillin.

Nutrient Solutions: The nutrient media contains serum-saline mixtures and various additives. For the most part, the ingredients and their concentrations have been empirically determined to support different types of explants and vary from tissue to tissue. That employed for spinal cord-DRG co-cultures consists of 33% human placental cord serum (heat inactivated at 56°C for 1/2 hour); 10% 9-day CEE; 50% Eagle's MEM plus glutamine; 7% BSS; 600 mg% of glucose; 1.3 ug/ml Achromycin and sterile tetracyclin HCL (Lederle NDC 005-4771-96).

Dissection and Maintenance of Organotypic cultures: All embryo dissections must be performed in sterile rooms with temperature-controlled air which has been filtered to remove particulate matter. When not in use, the rooms are swabbed down with 80% alcohol and exposed to ultraviolet irradiation. Dissection and maintenance of explant are performed by personnel wearing cap, mask, and full gown. To establish the nerve-muscle organotypic culture, 13-day-pregnant-mice (Swiss CD-1) are killed either by cervical dislocation or ether inhalation and soaked for 10 minutes in 70% ethyl alcohol. The animal is oriented on a cork dissection board and pinned ventral side up. Sterile instruments are used to reflect the skin, the

peritoneal cavity is entered and the uterine horns which contain the embryos are removed and placed in a petri dish containing tepid BSS. Working under a dissecting scope, the individual embryos are removed from the uterine sack and placed in the salt solution. The embryo's head and tail are removed using iridectomy scissors and the rib cage with accompanying viscera is trimmed away. The embryo is oriented dorsal side up and using fine forceps, the skin and muscle are separated from around the spinal vertebrae. A straight-edge scalpel blade is used to trim away any remaining tissues attached to the spinal cord. The remaining tissues are removed from the vertebrae with fine forceps and the perfectly intact spinal cord with its attached DRG is tranferred to a clean petri dish, dorsal side up. Using criss-cross motions with sterile straight-edge scalpel blades, slices of spinal cord with attached DRG are serially removed and held in fresh BSS. Care is taken that the dimension of the cord cross-sections do not exceed 0.5mm in thickness since nutrients and oxygen are supplied to the tissue by diffusion. After the dissection is performed, a Maximow chamber assembly is prepared by placing a 40mm square sterile coverslip on a background piece of black filter paper in large (150x20) sterile petri dish. The collagen coated coverslips are removed from their BSS storage fluid, drained, and placed face up on the square coverslip. Using curved forceps, individual cord sections are centered on the collagen substrate, allowed to settle and then fed with a single drop of nutrient medium to which 10-20 units/ml of nerve growth factor has been added. The Maximow chamber is pressed over the square coverslip and the explant is lifted out of the pertri dish and briefly gassed with 5% CO_2 to adjust the pH of the nutrient fluid. The square coverslip is sealed to the Maximow chamber with a paraffin-vaseline mixture and the whole assembly is placed in a specially designed wooden rack

which holds the Maximow slide in a horizontal lying-drop position. The cultures are constantly incubated at 34-35°C (see Fig. 1).

To establish nerve-muscle co-cultures, the Maximow chambers are opened after neuritic outgrowth has occured and a thin strip of striated muscle, removed from the hind-limb of an adult mouse, is positioned approximately 1mm away from the ventral cord outgrowth and allowed to settle into the collagen substrate. The culture is administered a fresh drop of nutrient fluid, and the complex is re-sealed in sterile Maximow chambers and incubated.

Twice a week, the cultures must be washed in BSS and fed a fresh drop of nutrient fluid. The wax seal around the Maximow chamber is broken and the collagen coverslip removed from the square coverslip with a needle and forceps, drained of exhausted nutrient fluid and washed for 5-15 minutes in tepid BSS. The culture is then replaced on clean square coverslip, administered a fresh drop of nutrient fluid and the culture is incorporated into a clean Maximow depression chamber.

Culture Development: At the time of muscle explantation, the spinal cord is relatively undifferentiated although the dorsal (sensory) and ventral (motor) horns and a central canal lined with ependymal cells can be identified (Fig. 2). Neuritic extension from the ventral cord first appears after 3-4 days in vitro (DIV). The paired DRG migrate away from the cord by 7-8 DIV. Spinal cord myelination begins after about 10 DIV. and proceeds over a period of several weeks. By 14-18 DIV, Schwann cells adopt a 1:1 relationship with axons and begin to myelinate peripheral nerve fibers. Adult skeletal muscle, incorporated into the system at 3-4 DIV, initially degenerates, then regenerates and differentiates, developing cross-striations and fibrillations by 14 DIV, features which depend upon

motor-fiber innervation for their prolonged viability. After 8 to 10 wk *in vitro*, the explants display a rich array of PNS myelinated sensory and motor fibers emanating respectively, from the dorsal and ventral horns of the spinal cord. Large anterior horn cells can be identified in the flattened peripheral regions of the ventral cord and a thick rim of CNS myelinated nerve fibers circles the cord's perimeter. Heavily myelinated PNS fibers present well-defined nodes of Ranvier and Schmidt-Laternman incisures. Sensory neurons within the paired DRG display large, centrally located nuclei with one or two nucleoli, and a fibrillar cytoplasm. The skeletal muscle, innervated by branches of large-diameter motor fibers, exhibit well-defined cross-striations and synchronous contractions. At maturity the cord-DRG-muscle organotypic culture, which measures only 5mm across, is a convincing facsimile of CNS-PNS neuromuscular tissue (Fig.3).

Put Figures 1-3 Here

Microscopy: The cultures can be removed from the incubator and examined by light microscopy for up to an hour with no apparent harm to the tissue. For microscopic examination and photography, objectives with long working distances are required to penetrate optically the Maximow chamber, the coverslips, the collagen gel, and the explant. For light and electron microscopy, cultures are removed from the Maximow chamber, immersed for 1 hr in cold, phosphate-buffered 2.5% glutaraldehyde, washed in Millonig's phosphate-buffer and postfixed for 1 hr in Dalton's 1% osmium tetroxide. Dehydration is achieved over a period of 70 min by immersing the collagen-coated coverslip with attached explant in increasing concentrations of ethanol. Dehydrated explants on their collagen substrate are gently scraped from the coverslip with a razor blade, cleared in propylene oxide and infiltrated with a 1:1 mixture of propylene oxide and epoxy resin for 30

min, followed by pure resin for 1 hr. The explant and collagen base is then sandwiched with a drop of resin between a pair of Teflon-coated 22mm square coverslips and placed in a 60°C oven overnight to polymerize the resin. This process produces a thin epoxy wafer which can be examined by bright-field or Nomarski-differentiated-contrast-interference microscopy and areas of interest are identified, and their position marked with a microscope-mounted etching device. One micrometer thick sections, stained with 1% Toluidine-blue, are examined by brightfield microscopy. Areas of interest can be trimmed from the epoxy wafer and mounted with a drop of resin onto a flat epoxy block which can then be sectioned and stained for electron microscopy. Organotypic sections examined by electron microscopy are ultrastructurally indistinguishable from in vivo tissues (Fig.4).

Put Figure 4 Here

Organotypic Model of Hexacarbon Neuropathy: The methodology described above was used to address both neuropathic and toxicologic inquires about hexacarbon neuropathy, a neurotoxic disease first recognized in Japan in the early 1960's through industrial accident (Wada et al., 1965; Yamada, 1964; Spencer et al., 1980). Workers in the laminating industry, exposed to solvents containing n-hexane, developed a central (CNS) and peripheral (PNS) polyneuropathy (i.e. sensory-motor), characterized by an insidious onset of muscle weakness, weight loss, and paresthesia in a "stocking-glove" pattern. With time, these symptoms progressed to a severe, debilitating, bilateral paralysis. Because of the widespread use of n-hexane and its related metabolite, methyl n butyl ketone (MnBK) in industrial settings, outbreaks of this neuropathy soon reached epidemic proportions, occurring in Italy (Rizutto et al., 1977), Morocco (Cavignaux, 1972), France (Gaultier et al., 1973), and the United States (Herskowitz et al., 1971; Allen et al., 1974;

Billmaier et al., 1974). In addition to occupational accident, prone individuals ("glue sniffers") who deliberately inhaled the vapors from solvents, lacquers, glue or glue thinners containing n-hexane (Prockop et al., 1974; Goto et al., 1974; Altenkirch et al., 1977) also developed the clinical symptoms described above.

Immediately following the outbreaks of hexacarbon neuropathy, a number of laboratories successsfully reproduced the disease in experimental animals (Spencer et al., 1975; Raleigh et al., 1975; Couri et al., 1977; Mendell et al., 1977). A salient neuropathic feature was the distal localization of nerve fiber degeneration and the preferential damage to the long and large ascending and descending CNS-PNS fiber tracts, in a classic "dying-back" fashion. A detailed study of hexacarbon-induced axonal degeneration, using individual teased nerve fibers from intoxicated rats (Spencer and Schaumburg, 1977a), suggested that the distal length of the axon and not its terminal was initially damaged, an observation that challenged the historical description of the "dying-back" pattern of neuropathy (Cavanagh, 1964). In its place the term "central-peripheral distal axonopathy" was coined to describe more accurately the neuropathy induced by hexacarbons (Spencer and Schaumburg, 1976).

Tantamount to this controversy were toxicological inquires as to the culpable neurotoxic hexacarbon metabolite. Clinical reports had indicated that both n-hexane and MnBK produced identical patterns of neurotoxic damage and it was proposed that the six metabolically interrelated hexacarbons identified by DiVincenzo and colleagues (1976, 1977), namely n-hexane, MnBK, 2-hexanol (2-hex), 2,5-hexanediol (2,5-HDiol), 5-hydroxy-2-hexanone (5-OH-2H) and 2,5-HD operated through a common metabolite (See Fig. 5).

Put Figure 5 Here

This notion gained support when investigators (Spencer and Schaumburg, 1975; Raleigh et al., 1975) demonstrated that 2,5-HD administered to experimental animals produced the characteristic pathology of hexacarbon neuropathy more rapidly than the parent compounds. Later, Krasavage and colleagues (1980) determined that the onset of functional and structural changes was related to serum levels of 2,5-HD. It soon became apparent that an *in vitro* system might aid in clarifying primary and relative neurotoxicity among hexacarbon metabolites since in the animal, hepatic biotransformation converted the parent compounds *n*-hexane and MnBK into an extensive range of minor inter-related metabolites (DiVincenzo et al., 1976). Although a variety of *in vitro* techniques was considered, the organotypic culture system was chosen because of its morphological parallels to *in situ* tissue counterparts. In general, the use of intact, mammalian tissues to model the neuropathy promised to yield the most directly relevant data to contemporaneous animal studies. The combination of fetal spinal cord with attached DRG, co-cultured with striated muscle, represented a morphological and physiologically-relevant model of the disease since the motor-sensory reflex arc had been indicated clinically as highly vulnerable to hexacarbon insult (Allen et al.,1974; Billmaier et al., 1974). For toxicity inquires, tissue culture afforded direct manipulations of the tissue's environment. Individual hexacarbon metabolites would be dissolved in the nutrient fluids, guaranteeing a direct and continuous exposure of test substance to the tissues. Although the organotypic co-cultures would be isolated from full-scale hepatic activity, chick embryo extract contained in the nutrient media would contribute sufficient microsomal activity for the neurotoxic activation of the parent hexacarbons, *n*-hexane and MnBK. Several features of the organotypic technique promised to aid in assessing both the primary

and relative neurotoxicity among the hexacarbons. The nutrient media, acting as the exposure vehicle for the individual metabolites, could be analyzed by gas liquid chromatography (GLC) to identify any conversion products. Since the pharmacological consideration of lipid-aqueous partitioning is minimized in tissue culture, a more accurate quantitation of applied versus absorbed compound could be made to aid in assessing relative toxicity among the metabolites. In addition, hepatic transformation, which effectively alters the concentration of test compounds by converting them to intermediate metabolites, would be by-passed.

Organotypic and dissociated ganglia cultures had been used in previous experiments to address the neurotoxicity of a range of chemicals including methyl mercury (Kim, 1971), thallium (Hendelman, 1969; Spencer, et al., 1973; Yonezawa et al., 1977), triethyltin (Grahamet al., 1975), clioquinol (Yonezawa et al., 1977), chloroquine (Tischner, 1972), colchicine (Peterson and Bornstein, 1968), and aluminum (Seil and Lampert, 1969). Encouraged by this history and by the unique features of organotypic methodology, studies were designed not only to clarify the neuropathic and neurotoxic ambiguities of hexacarbon neuropathy but also to evaluate critically the overall validity of using this culture system to model a human toxic neuropathy (Veronesi, 1980; Veronesi et al., 1980, 1983).

The individual hexacarbon metabolites, namely, n-hexane, 2-hex, 2,5-HDiol, 5-OH-2H, MnBK and 2,5-HD were exposed to mature nerve-muscle cultures (n=30-50/metabolite). Cultures treated with the diketone 2,4-hexanedione (2,4-HD), a non-neurotoxic hexacarbon *in vivo* (Spencer et al., 1978), acted as negative controls and age-matched cultures exposed to regular nutrient fluid were used as normal controls. Each test compound was dissolved in BSS to a stock concentration and after Millipore-filtration,

dissolved in nutrient fluid in test concentrations. This fluid was administered to the cultures twice weekly under sterile conditions. To qualify as an in vitro model of animal hexacarbon neuropathy it was critical that axonal damage should begin only after weeks of exposure, and that fiber degeneration should develop slowly so that the individual stages could be observed, described and related to the in vivo neuropathological process. Mature explants were treated with 50-600 ug/ml of each hexacarbons dissolved in nutrient fluid. High (400-650 ug/ml) levels of each hexacarbon induced pancytotoxic damage and cord necrosis within days, while the lower doses (50-100 ug/ml) failed to produce pathology after several months of exposure. Continous exposure to 245-325 ug/ml of each metabolite (except 2,4-HD), however, produced the following sequence of pathogenic change (Fig. 6).

Put Figure 6 Here

After 3-6 weeks of exposure (depending on the individual metabolite) the nodes of Ranvier in distal lengths of nerve fibers to elongate. Several days later, focal paranodal axonal swellings developed proximal to the elongated nodal region. These swellings increased in size with continued exposure sometimes reaching 2-3 times the original fiber diameter. They characteristically developed on the proximal face of one or more elongated nodes of Ranvier, in the distal regions of large-diameter sensory and motor axons, and often involved the terminal node of Ranvier in the stem of a branching motor fiber (Fig. 7). Electron microscopy of these axonal swellings revealed them to be ultrastructurally identical to that seen in vivo (Spencer and Schaumburg, 1977a). Cross sections taken through paranodal regions of focally swollen axons displayed excessive 10 nm neurofilaments, peripherally displaced groups of microtubules, disrupted mitochondria and a disproportionately thin or absent myelin sheath (Fig. 8). With continued

intoxication, paranodal swellings developed more proximally along the affected nerve fibers, involving more successive nodes of Ranvier. With time the myelin sheath surrounding these swellings slowly retracted and became atypically refactile under brightfield microscopic examination. The myelin slippage continued and often resulted in internodal demyelination (Fig. 9).

Put Figure 7-9 Here

Subsequent to the development of the giant axonal swellings, the fiber length distal to it would shrink in diameter and after time, degenerate in a Wallerian-like fashion. This sequence (viz. nodal elongation, axonal swelling, myelin retraction, distal fiber attenuation and degeneration), would subsequently repeat itself at more proximal lengths along the affected fibers giving an overall effect of "dying-back." In response to this degeneration, muscle tissue would lose its well-defined striated pattern and cease to contract. In several cultures, the explant was allowed to "recover" in plain nutrient media for several weeks (Fig. 10). Denuded axonal swellings located at the distal lengths of damaged nerve fibers were seen to return to normal fiber diameter and several weeks later become re-ensheathed with thin layers of myelin. In this fashion, living peripheral fibers responded to hexacarbon intoxication, and supported the pattern of distal "dying-back" axonopathy that had been previously inferred from <u>in vivo</u> studies (Spencer and Schaumburg, 1977 a,b,).

Put Figure 10 Here

The metabolic fate of individual hexacarbon metabolites was examined by removing the "exhausted" test fluid from individual cultures at the time of feeding and submitting it for GLC analysis. These data (Table 1) indicated that all cultures converted their individual test metabolites to 2,5-HD, which in itself was neurotoxic in the absence of metabolic conversion. In

addition, these data indicated that the organotypic explants were metabolically competent to perform the w-1 oxidation-reduction reactions that had been postulated (Frommer et al., 1974; Kramer et al., 1974) to yield the six major interrelated metabolites noted in vivo (DiVincenzo, 1976, 1977).

The question of primary neurotoxicity was examined by ranking the metabolites according to neurotoxic potency since the potency of a primary toxic metabolite is greater than that of its precursor compounds. Age-matched cord-ganglion cultures, with identical feeding and washing schedules, were exposed to equimolar (2.8mM) concentrations of the individual metabolites (n=12/metabolite) and the time-delay for the appearance of definitive axonopathic change (viz. axonal swelling with accompanying myelin retraction) was recorded (Table 2). An order of neurotoxic potency was assembled (i.e., 2,5-HD=MnBK > 5-OH-2H > 2,5-HDiol > n-hexane > 2-hex) that indicated that the further removed a compound was metabolically from the common neurotoxic metabolite 2,5-HD, the less neuropathic it was in culture. An exception to this was the equivalent neurotoxic potency of MnBK which had been examined in four separate trials involving a total of 60 additional cultures. Earlier investigators (Abdel-Rahman et al., 1976) had also reported the equipotent neurotoxicity of MnBK in animals. GLC analysis of our test lot of MnBK which had been scored as 97% pure revealed a 2.7% contamination with methyl iso-butyl ketone, a hexacarbon which has recently been shown to potentiate hexcarbon neuropathy by inducing the P450 system (Abou-Donia et al., 1985).

Although other in vitro systems were used to evaluate hexacarbon neurotoxicity (Goldstein, 1977; Tsukamoto and Yonezawa, 1978; Selkoe et al., 1978), the experiments described above made significant contributions to understanding the disease since they paralleled animal studies with

respect to target tissue, duration of exposure and neuropathic end-points. The unique features of the organotypic model permitted the sequence of hexacarbon-induced nerve degeneration and repair to be assembled, and identified 2,5-HD as the common, possibly primary, neurotoxic hexacarbon. Based on the success of these experiments, the model was later used to replicate more sophisticated neurotoxic interactions involving the potentiation of hexacarbon neuropathy by methyl ethyl ketone (Veronesi et al., 1984; Veronesi, 1984). In effect, the success of the above experiments, as a series, secured the use of tissue culture for future neurotoxic modeling.

Table 1

Metabolic Fate of Hexacarbon Metabolites[1]

Test Compound (ug/ml)	GLC Assayed Compounds					
	n-Hexane	2-Hex	2,5-HDiol	MnBK	5-OH-2Hex	2,5-HD
n-Hexane (245)	+		+			+
2-Hexanol (290)		+			+	+
2,5-HDiol (320)						+
MnBK (280)		+	+	+		+
5-OH-2-H (320)			+		+	+
2,5-HD (320)						+
2,4-HD (320)						-

Metabolic Fate: Samples of exhausted nutrient fluids were removed from each hexacarbon-treated culture group, pooled according to test group, stored at 20°C in sealed GLC microvials (Supelco No. 3-3208) and shipped in dry ice for collaborative GLC analysis[1].

1. Performed by George DiVincenzo, Eastman Kodak, Rochester, NY.

Table 2
Relative Neurotoxic Potency Among Hexacarbon Metabolites

Hexacarbon	Concentration (ug/ml) 2.8 mM	Onset of Myelin Retraction (days)
n-Hexane	245	29
2-Hexanol	290	30
2,5-HDiol	325	27
MnBK	280	15
5-OH-2Hex	325	26
2,5-HD	325	15

Relative Neurotoxicity Age-matched cord-ganglion cultures were exposed to equimolar (2.8mM) concentrations of individual hexacarbon metabolites. The time delay for the appearance of pathological changes (axonal swellings and myelin retraction) was recorded for each set of cultures and used to establish a temporal sequence of relative toxicity.

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FIGURES

Figure 1. Diagram of organotypic nerve-muscle methodology. Explants of spinal cord, DRG and striated muscle are grown on collagen-coated, circular coverslips (top), placed on square coverslips and fed a drop of nutrient media. They are then inverted, incorporated into Maximow chambers, sealed with a paraffin-vaseline mixture, and incubated.

Figure 2. Spinal cord section with attached DRG after 3 DIV. Brightfield; living tissue. x65.

Figure 3. Mature organotypic culture consisted of spinal cord explant and attached DRG. Note sensory and motor fibers emerging from dorsal and ventral horns, respectively. (Photograph courtesy of ER Peterson). Brightfield, living culture x65.

Figure 4. Electron micrograph of PNS fascicle from control culture displays ultrastructural similarity to <u>in vivo</u> tissues. Electron micrograph x9600.

Figure 5. The major metabolites of the parent hexacarbon compounds n-hexane and MnBK are shown.

Figure 6. Cultures exposed to 2.8 mM MnBK for 3-8 weeks. (A) Nodal (n) elongation, an early pathological change seen in large diameter motor and sensory fibers of intoxicated cultures. (Normaski; fixed culture, X1,375). (B) Paranodal axonal swelling (*) develops on the proximal side of a node of Ranvier (n) (Normaski; fixed culture x3750). (C). Myelin retracts around the swollen axon leaving a denuded axon (Brightfield; living culture, x1200). (D) Fiber distal to the swelling attenuates and eventually degenerates in a Wallerian-like fashion (Brightfield; living culture, x1200).

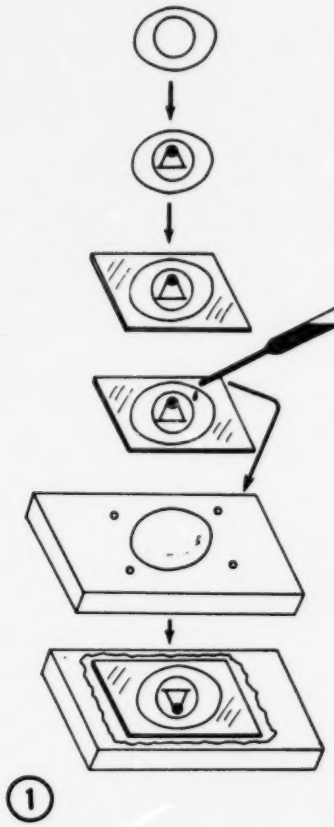
Figure 7. Motor nerve fiber from a 2,5-HD-treated explant displays a proximal paranodal axonal swelling at the site of bifurcation. Whole mount, Holmes silver stain, Brightfield; x65.

Figure 8. Cross-section of a paranodal axonal swelling showing massive accumulations of neurofilaments and a disproportionally thin myelin sheath as a consequence of retration. Culture treated with MnBK X22,000 (Photograph courtesy of PS Spencer).

Figure 9. The paranodal region of two distal internodes is shown. The proximal paranodal axon is swollen with neurofilaments and is completely denuded of myelin. The distal paranodal fiber is relatively normal. Culture treated with 2,5-HD for 6 weeks. X12,500.

Figure 10. A paranodal axonal swelling is noted in the distal end of a large diameter fiber(1). Myelin retraction begins after 1-2 weeks (2) and continues leaving a denuded paranodal swelling (3). The culture is returned to plain nutrient fluid and maintained for several weeks. During this time, the swelling undergoes reconstitution (viz. returns to

normal dimensions) and new myelin is laid down (arrow 5). Culture treated for 12 weeks with 5-OH-2Hex Brightfield, living tissue X500).



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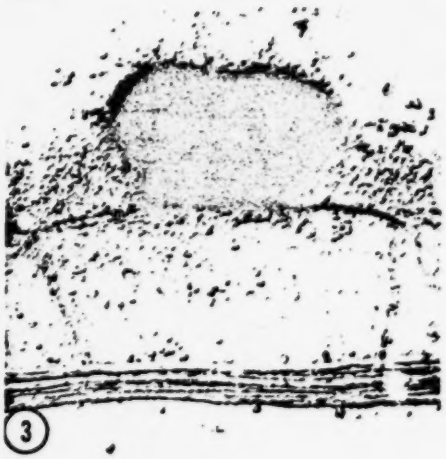

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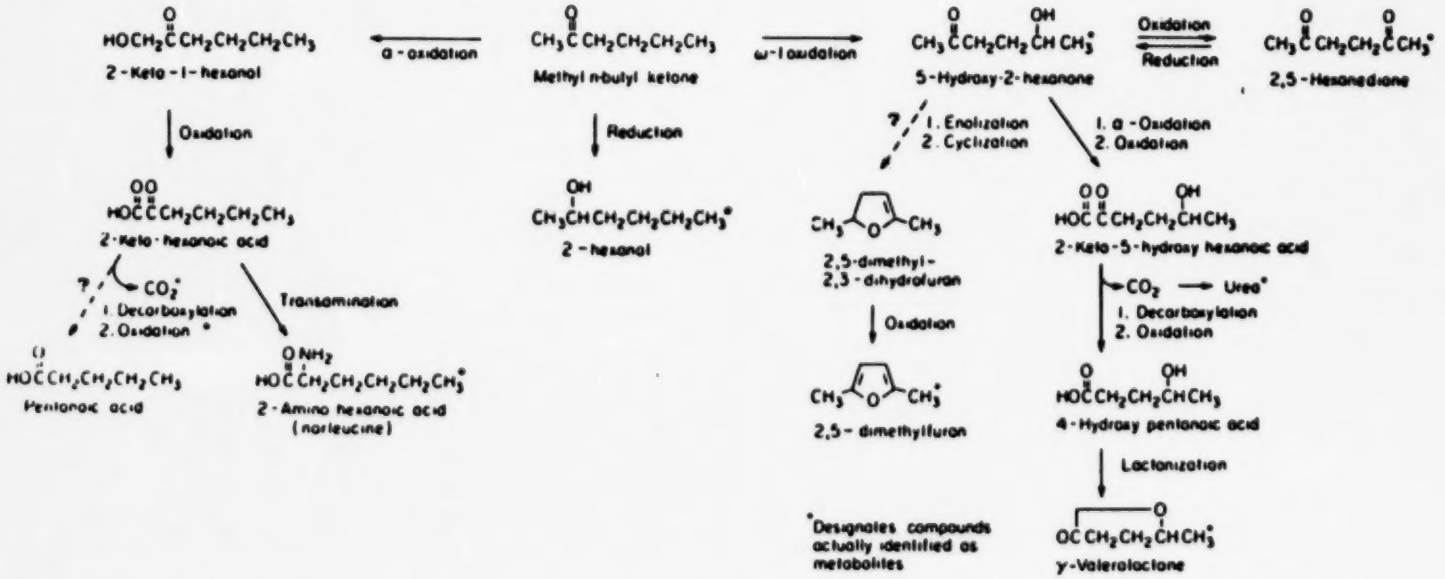
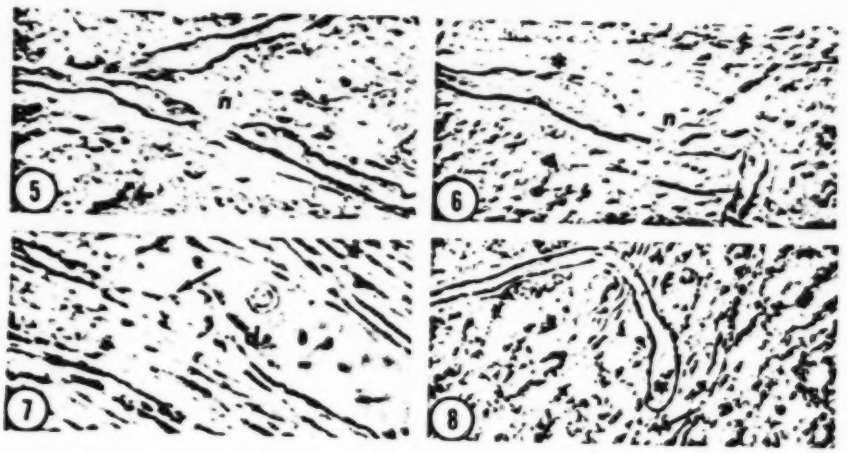

2-Keto-1-hexanol
α-oxidation
Methyl n-butyl ketone
ω-1 oxidation
5-Hydroxy-2-hexanone
Oxidation
Reduction
2,5-Hexanedione
Oxidation
Reduction
1. Enolization
2. Cyclization
1. α-Oxidation
2. Oxidation
2-Keto-hexanoic acid
2-hexanol
2,5-dimethyl-2,3-dihydrofuran
2-Keto-5-hydroxy hexanoic acid
1. Decarboxylation
2. Oxidation
Transamination
Oxidation
Urea*
Pentanoic acid
2-Amino hexanoic acid (norleucine)
2,5-dimethylfuran
4-Hydroxy pentanoic acid
Lactonization
*Designates compounds actually identified as metabolites
γ-Valerolactone

5

(6)



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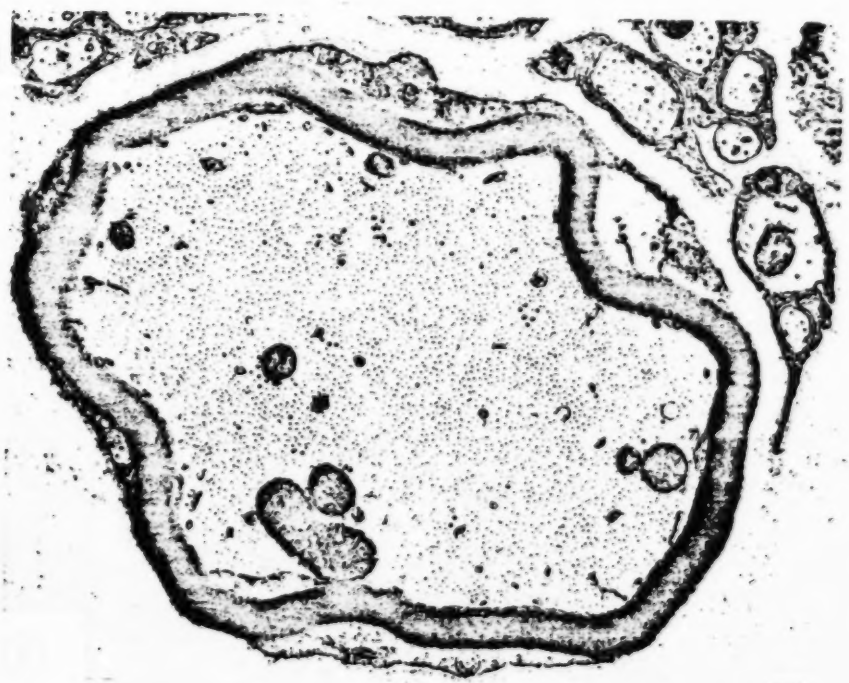

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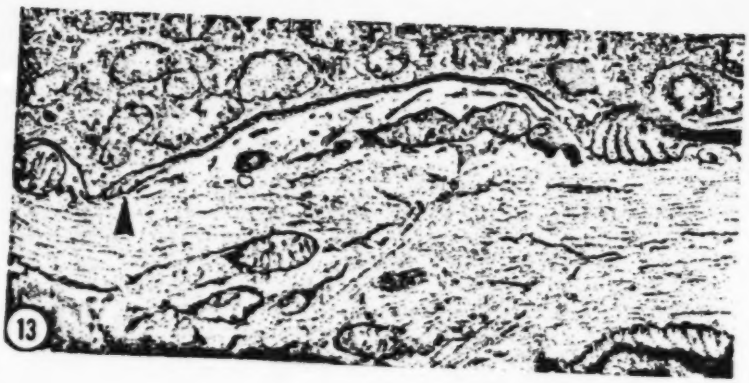

(10)